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1. **Holland et al. Anal. Biochem. 1994, 222(2), pp. 516-518.**
2. **Peterson et al. Anal. Biochem. 1999 (7/1/99), 271(2), pp. 131-136.**
3. **Antonsson et al. Anal. Biochem. 1999 (2/15/99), 267(2), pp. 294-299.**
4. **Park et al. Anal. Biochem. 1999 (4/10/99), 269(1), pp. 94-104.**

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The Use of β,γ-Methyleneadenosine 5′-Triphosphate to Determine ATP Competition in a Scintillation Proximity Kinase Assay

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A novel method for characterizing the kinetics of protein kinase inhibitors is described. This method uses glycogen synthase kinase β as the model protein kinase and looks at the shift in IC_{50} of inhibitors using the nonhydrolyzable ATP analog, β,γ-methyleneadenosine 5′-triphosphate, also known as AMP-PCP. Due to its inability to be hydrolyzed, AMP-PCP is being used to characterize known glycogen synthase kinase inhibitors by determining the shift in IC_{50} at concentrations above its calculated K_i of 490 μM. The assay format for the detection of inhibition is a scintillation proximity assay which is robust and reproducible at very low levels of [γ-^{33}P]ATP. The use of AMP-PCP coupled with the use of the scintillation proximity assay allows this characterization of inhibition without increasing [γ-^{33}P]ATP and without significantly diluting the overall assay signal. We have used this method in kinetic analyses to demonstrate that we can detect a significant shift in IC_{50} with the known ATP competitive inhibitors, staurosporine, Ro 31-8220, and olomoucine. The IC_{50} for glycogen synthase peptide and lithium chloride, which has been reported to be uncompetitive, remains unchanged. © 1999 Academic Press

Glycogen synthase kinase 3 (GSK3)[2] was first discovered as a skeletal muscle serine/threonine kinase that was capable of phosphorylating and inhibiting the activity of glycogen synthase (1). GSK3, itself, is inhibited in response to insulin, which has been postulated to contribute to the stimulation of glycogen synthesis (2). The upstream regulator of GSK3 has been shown to be PKBα, which is also a serine/threonine kinase that phosphorylates GSK3α and GSK3β on Ser-21 and Ser-9, respectively (3). In addition to phosphorylating glycogen synthase, GSK3 has been shown to phosphorylate a number of other proteins such as c-*jun* (4, 5), CREB (cAMP response element binding protein) (6), inhibitor 2 (7), ATP citrate lyase (8), τ protein (9), and IRS-1 (insulin receptor substrate 1) (10). Activity of GSK3 toward some of these substrates requires priming by prior phosphorylation of proximal residues via another kinase. In the case of GSK3 activity toward glycogen synthase, the serine/threonine kinase, casein kinase II, is required to phosphorylate a proximal serine residue (11). GSK3 has two isoforms, α and β, and both of these isoforms recognize the motif Ser-X-X-X-pSer, such as is found in the glycogen synthase peptide used in our assay, Ser-Arg-His-Ser-Ser-Pro-His-Gln-pSer-Glu-Asp-Glu-Glu-Glu.

Measurement of serine/threonine kinase activity is often done by conventional incorporation of radiolabeled ATP into a substrate protein or a peptide due to the accuracy and simplicity of the assay and also frequently due to the lack of appropriate phospho-specific antibodies. Radiometric assays, however, can be limiting when used to determine the kinetic characteristics of inhibitors. For example, an easy technique to determine if a compound is kinetically competitive with ATP is to measure the IC_{50} of the inhibitor at several concentrations of substrate above and below the K_m for that substrate. If the inhibitor is kinetically competitive with substrate, the IC_{50} will be increased as described by the Cheng–Prusoff equation (12). In a radiometric assay, however, the substrate, ATP, would have to be labeled to very high specific activity, which is often not practical, especially when dealing with numerous inhibitors.

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[2] Abbreviations used: GSK3, glycogen synthase kinase 3; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; SPA, scintillation proximity assay.

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In this paper, we describe the use of the scintillation proximity assay (SPA, 13) in conjunction with the nonhydrolyzable ATP analog, β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP), to characterize known GSK3β inhibitors. AMP-PCP, by nature an ATP-competitive inhibitor, should cause a shift in the IC_{50} of those inhibitors that bind at the ATP site. The IC_{50} of inhibitors that bind at a site other than the ATP site should remain unchanged. As predicted, we show that the IC_{50}'s of known ATP-competitive inhibitors, staurosporine, olomoucine, and Ro 31-8220, are strongly shifted by AMP-PCP while glycogen synthase peptide and LiCl are minimally affected. The novelty of this method lies in the efficiency of the SPA at very low levels of radioactivity and the ability of AMP-PCP to measurably shift the IC_{50}.

MATERIALS AND METHODS

Materials. The following reagents were used: GSK3β and the glycogen synthase peptide (Upstate Biotechnology); biotinylated glycogen synthase peptide, biotin-6C-Ser-Arg-His-Ser-Ser-Pro-His-Gln-pSer-Glu-Asp-Glu-Glu-Glu-OH (Research Genetics, Inc.); AMP-PCP, staurosporine, olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine), and lithium chloride (LiCl) (Sigma); Ro 31-8220 (3-(1-(3-(amidinothio) propyl-1H-indol-3-yl)-3-(1-methyl-1H-indol-3-yl) maleimide methane sulfonate) (Calbiochem); SPA polyvinyl toluene beads and [γ-^{33}P]ATP (Amersham); and opaque, clear-bottomed microtiter plates (Wallac).

GSK3β scintillation proximity assay. The biotinylated glycogen synthase peptide was added at a final concentration of 1.0 μM in a 120-μl assay buffer containing 8.0 mM morpholinepropanesulfonic acid (pH 7.0), 1.0 mM dithiothreitol, 2.0 μM ATP, and the indicated concentration of AMP-PCP. The inhibitors, staurosporine, olomoucine, Ro 31-8220, LiCl, or unbiotinylated glycogen synthase peptide, were then added and the reaction was initiated by the addition of 45 mU of GSK3β and 0.02 μCi/well of [γ-^{33}P]ATP. After a 75 min incubation at room temperature, each reaction was stopped by the addition of a buffer containing a final concentration of 5.0 mM EDTA, 50 μM ATP, 0.1 % Triton X-100, and 0.5 mg streptavidin polyvinyl toluene beads. The streptavidin beads were allowed to settle for 16 h and the plates were counted on a Wallac Trilux Microbeta counter.

RESULTS AND DISCUSSION

Our GSK3β assay uses concentrations of ATP and peptide, the two substrates in this reaction, that are well below their respective K_ms of 20 μM (1) and 5 μM (14). This enables us to detect inhibitors that are competitive at either of the substrate binding sites. We also wanted a quick way to be able to distinguish those inhibitors that were competing at the ATP site; however, addition of a concentration of ATP required to see a significant shift in the IC_{50} of an ATP competitive inhibitor would cause the signal to be too dilute in a kinase reaction containing only 0.02 μCi of [γ-^{33}P]ATP (see Materials and Methods). Therefore, we decided to run the assay in the presence of a known ATP-competitive, nonhydrolyzable ATP analog, AMP-PCP, instead of dramatically increasing the specific activity of ATP. AMP-PCP has an IC_{50} of 460 μM (Fig. 1), and the calculated K_i is approximately the same value since the 2 μM concentration of ATP in our assay is well below its K_m of 20 μM (1). If the IC_{50} values for other ATP competitive inhibitors are determined in the presence of above K_i levels of AMP-PCP, there will be a concurrent-fold shift in activity (see Fig. 2A). This shift is described by a modified Cheng–Prusoff equation for multiple inhibitors (12), $IC_{50} = 1 + K_eX/(S + K_e)K_x$, where the IC_{50} for an inhibitor is measured in the presence of different concentrations of AMP-PCP. In this equation, X is the concentration of AMP-PCP and S is the concentration of the substrate, ATP. Under the conditions of our assay, $S \ll K_e$ and the equation simplifies to $IC_{50} = 1 + ([X]/K_X)$.

To test the utility of this approach, we chose to look at staurosporine, olomoucine, Ro 31-8220, LiCl, and the unbiotinylated glycogen synthase peptide. The first three compounds are expected to behave in an ATP competitive manner according to the literature or the structure in the case of Ro 31-8220 (15–19). The pep-

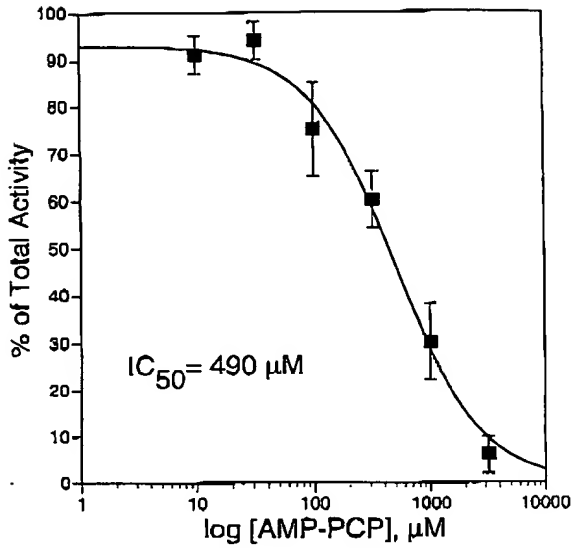

FIG. 1. Inhibition of GSK3β by AMP-PCP. GSK3β (45 mU) was incubated with 1.0 μM peptide, 2.0 μM ATP, and the indicated concentrations of AMP-PCP as described under Materials and Methods. The resulting IC_{50} of 490 μM is indicated and the data are representative of two experiments performed in triplicate.

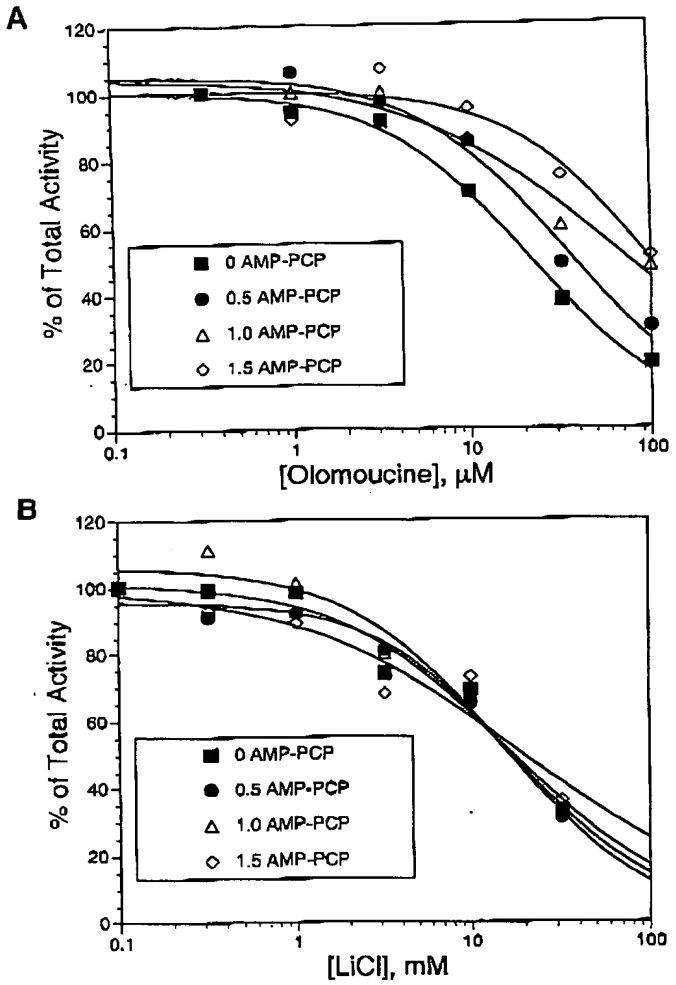

FIG. 2. Effect of AMP-PCP on the % activity of GSK3β at different concentrations of AMP-PCP and (A) olomoucine or (B) LiCl. 1.0 μM peptide, 2.0 μM ATP, and the indicated concentrations of AMP-PCP were incubated with olomoucine or LiCl, and the reaction was initiated by the addition of [γ-^{33}P]ATP and GSK3β. The reaction was stopped in a buffer containing SPA beads as described under Materials and Methods and, after 16 h, was counted on a Wallac Trilux Microbeta counter. Data are representative of two experiments done in triplicate at six indicated concentrations of inhibitor.

tide, by nature, should behave noncompetitively and LiCl is believed to be uncompetitive according to a recent report (20).

Staurosporine is a microbial alkaloid that was initially described as having an IC_{50} against protein kinase C of 2.7 nM (21). Since then, staurosporine has also been tested aginst numerous other protein kinases (22) and has been found to be relatively nonselective with IC_{50}s in the nanomolar range for serine/threonine kinases such as protein kinase A (23), phosphorylase kinase (23), S6 kinase (23), cdc2 (22), and CDK2 (24), as well as the tyrosine kinases, v-Src (25) and Lyn (22). There is some selectivity, however, as staurosporine is not as potent an inhibitor against some kinases such as ERK-1 with an IC_{50} of 1.5 μM (21) and casein kinases 1 and 2 with IC_{50}s of 164 and 19.5 μM, respectively (22). Recently, the structure of CDK2 complexed with staurosporine was solved to 2-Å resolution (15). The complex provided physical evidence that the binding of staurosporine to CDK2 occurs at the active site. Using SPA with GSK3β, we have also found that staurosporine is a potent inhibitor with an IC_{50} of 28 nM (Fig. 3A). Addition of increasing concentrations of AMP-PCP from 0 to 1.5 mM caused a shift in the IC_{50} from 28 to greater than 100 nM, indicating that staurosporine is competitive with ATP at the active site of GSK3β.

Also initially demonstrated as a protein kinase C inhibitor, Ro 31-8220 is a staurosporine analog that has been found to inhibit numerous other kinases (16). Ro 31-8220 markedly inhibited MAPKAP-K1β kinase and S6 kinase with IC_{50}s of 3 and 15 nM, respectively. This analog also inhibited PKBα with a less potent IC_{50} of 1.0 μM and was completely inactive against MAPKAP-K2, c-Raf1, MAPKK-1, and p42 MAP kinase (16). We looked at the effect of Ro 31-8220 on GSK3β and

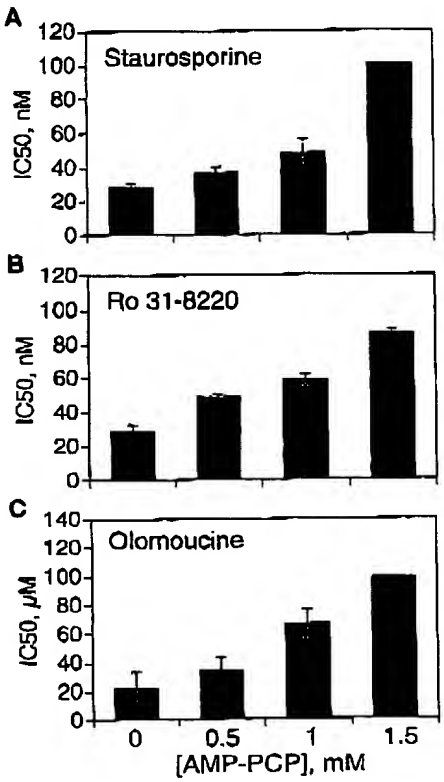

FIG. 3. Effect of AMP-PCP on the IC_{50} of known, competitive inhibitors. The assay was done as described in the legend to Fig. 2 except with the addition of (A) staurosporine, (B) Ro 31-8220, or (C) olomoucine. Data are representative of two experiments done in triplicate at six different concentrations of inhibitor.

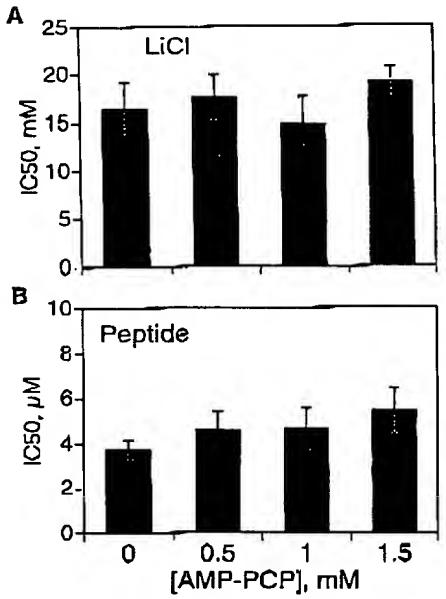

FIG. 4. Effect of AMP-PCP on the IC_{50} of known, noncompetitive inhibitors. The assay was done as described in the legend to Fig. 2 except with the addition of (A) LiCl or (B) peptide. Data are representative of two experiments done in triplicate at six different concentrations of inhibitor.

found that this analog potently inhibited activity with an IC_{50} of 29 nM. The addition of 1.5 mM AMP-PCP caused a shift in the IC_{50} to 82 nM (Fig. 3B).

Olomoucine was first discovered as a plant cytokinin derivative that appeared to inhibit protein kinases and was subsequently shown to have some potency and selectivity for proline-directed, serine/threonine kinases albeit in the micromolar range (17). Cdc2, CDK2, CDK5, and ERK-1 are inhibited by olomoucine with IC_{50} values of 7, 7, 3 and 25 μM, respectively. A physiological GSK from *S. cerevesiae* also has a reported IC_{50} of 100 μM for olomoucine (18) and we have found an IC_{50} value with rabbit GSK3β of 22 μM (Fig. 3C). In both cdc2 (17) and GSK3 (18), olomoucine has been found to be competitive with respect to ATP, and the crystal structure of olomoucine complexed with CDK2 demonstrates binding within the ATP pocket (19). As with staurosporine and Ro 31-8220, the IC_{50} of olomoucine was shifted dramatically with AMP-PCP and increased from 22 to 98 μM at the highest concentration of AMP-PCP (Figs. 2A and 3C).

The last two inhibitors that we examined are not competitive with ATP. The nonbiotinylated glycogen synthase peptide would not be expected to compete with ATP, while LiCl has been described as uncompetitive (20). Our data corroborate this as LiCl (Figs. 2B and 4A) and the peptide (Fig. 4B) showed essentially no change in IC_{50} with the addition of AMP-PCP, even at the highest concentration of 1.5 mM.

According to the previously described modified Cheng–Prusoff equation (12) for multiple inhibitors where the $IC_{50} = 1 + ([AMP\text{-}PCP]/K_i)$, there should be a 4.1-fold increase in the IC_{50} of compounds that are competitive with ATP when the concentration of AMP-PCP equals 1.5 mM and it is assummed that the K_i equals 490 μM. It can quickly be seen in Fig. 5 that only staurosporine, Ro 31-8220, and olomoucine approach this 4.1-fold change, while LiCl was unchanged and the peptide showed very little change.

We also did a more in-depth kinetic analysis on the representative inhibitors, olomoucine and LiCl. For olomoucine, the derived equation for two competitive, exclusive inhibitors is $1/v = (K_s/K_iV_{max}[S])[I] + 1/V_{max}(1 + K_s/[S] + K_x[X]/[S]K_x)$, where I is the inhibitor (26). A Dixon plot of $1/v$ versus olomoucine at a fixed concentration of AMP-PCP and ATP should be linear with a slope of $K_s/[S]V_{max}K_i$. If both olomoucine and AMP-PCP are competitive with respect to ATP and are mutually exclusive, the curves at different concentrations of AMP-PCP should be parallel since the slope is independent of AMP-PCP . As can be seen in Fig. 6A, the lines are indeed parallel at different concentrations

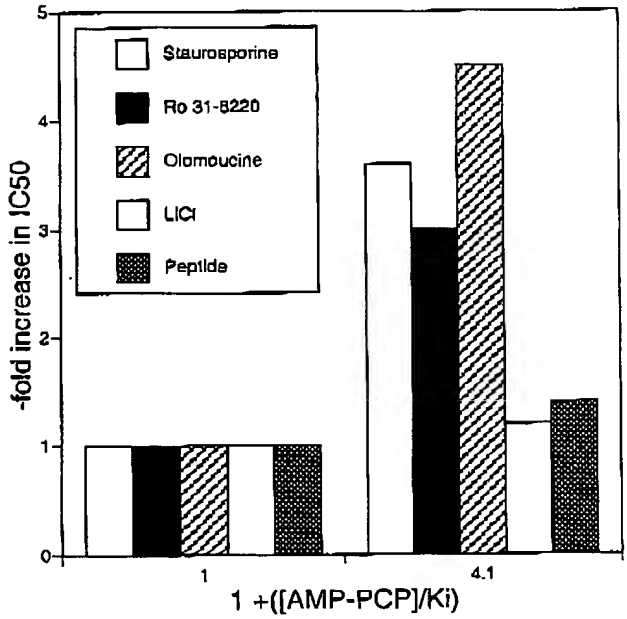

FIG. 5. Overall increase in IC_{50} of inhibitors at 1.5 mM AMP-PCP. The assay was done as described in the legend to Fig. 2 and the data are plotted as $1 + ([AMP\text{-}PCP]/K_i)$ vs -fold increase in IC_{50} for all of the inhibitors. According to this equation, $1 + ([AMP\text{-}PCP]/K_i)$, the ratio of the IC_{50} in the presence of AMP-PCP to the IC_{50} in the absence of AMP-PCP should equal 1 when the [AMP-PCP] is 0 mM and its K_i is 490 μM. When the [AMP-PCP] equals 1.5 mM, the ratio should be 4.1.

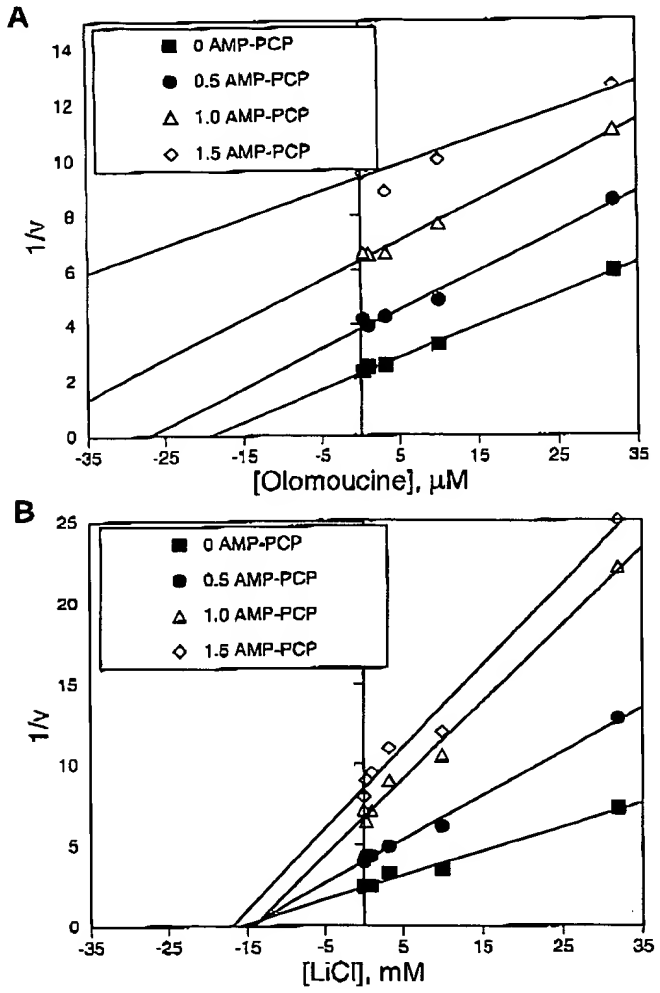

FIG. 6. Dixon plots of $1/v$ versus (A) olomoucine and (B) LiCl at different, fixed concentrations of AMP-PCP and a constant ATP. The parallel plots in (A) show that olomoucine and AMP-PCP are competitive, whereas the intersecting plots in (B) indicate that LiCl and AMP-PCP are noncompetitive.

of AMP-PCP. In addition, the K_i value of 22 μM from the the x-intercept when the concentration of AMP-PCP equals zero compares with our experimental value of 22 μM as the IC_{50} for olomoucine (Fig. 3C).

For LiCl as an inhibitor, a Dixon plot of i/v versus [LiCl] at fixed concentrations of AMP-PCP and ATP was dependent on the concentration of AMP-PCP, and the slope increased as AMP-PCP increased with a point of intersection on the x-axis equal to $-K_i$ (Fig. 6B). Our analysis fits the derived equation for a mixture of a nonexclusive, competitive, and a noncompetitive inhibitor, $1/v = K_a/V_{max}[S](1/K_i + [S]/K_iK_a + [X]/K_xK_i)[I] + 1/V_{max}[S](K_a + [S] + [X]K_a/K_x)$ (26), despite the literature report of LiCl as an uncompetitive inhibitor (20).

The K_i from Fig. 6B is approximately 16 μM, which, in fact, is in close agreement with our own experimental value of 16 μM (Fig. 4A).

Taken together, the above results show the utility of using AMP-PCP to determine the competitive nature of inhibitors. The addition of AMP-PCP will not significantly dilute the overall signal when using a robust and reproducible assay such as SPA. This novel method will prove especially useful under conditions where numerous assays are performed and it is necessary to keep the concentration of [γ-^{33}P]ATP at reasonable levels.

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REFERENCES

1. Embi, N., Rylatt, D. B., and Cohen, P. (1980) *Eur. J. Biochem.* **107**, 519–527.
2. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) *EMBO J.* **14**, 4288–4295.
3. Cross, D. A. E., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature* **378**, 785–789.
4. Nikolakaki, E., Coffer, P. J., Hemelsoet, R., Woodgett, J. R., and Defize, L. H. K. (1993) *Oncogene* **8**, 833–840.
5. de Groot, R. P., Auwerx, J., Bourouis, M., and Sassone-Corsi, P. (1993) *Oncogene* **8**, 841–847.
6. Fiol, C. J., Williams, J. S., Chou, C.-H., Wang, Q. M., Roach, P. J., and Andrisani, O. M. (1994) *J. Biol. Chem.* **269**, 32187–32193.
7. Vandenheede, J. R., Yang, S.-D., Goris, J., and Merlevede, W. (1980) *J. Biol. Chem.* **255**, 11768–11774.
8. Hughes, K., Ramakrishna, S., Benjamin, W. B., and Woodgett, J. R. (1992) *Biochem. J.* **288**, 309–314.
9. Sperber, B. R., Leight, S., Goedert, M., and Lee, V. M.-Y. (1995) *Neurosci. Lett.* **197**, 149–153.
10. Eldar-Finkelman, H., and Krebs, E. G. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 9660–9664.
11. Fiol, C. J., Wang, A., Roeske, R. W., and Roach, P. J. (1990) *J. Biol. Chem.* **265**, 6061–6065.
12. Cheng, Y.-C., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22**, 3099–3108.
13. Baum, E. Z., Johnston, S. H., Bebernitz, G. A., and Gluzman, Y. (1996) *Anal. Biochem.* **237**, 129–134.
14. Woodgett, J. R. (1989) *Anal. Biochem.* **180**, 237–241.
15. Lawrie, A. M., Noble, M. E. M., Tunnah, P., Brown, N. R., Johnson, L. N., and Endicott, J. A. (1997) *Nature Struct. Biol.* **4**, 796–801.
16. Alessi, D. R. (1997) *FEBS Lett.* **402**, 121–123.
17. Vesely, J., Havlicek, L., Strnad, M., Blow, J. J., Donella-Deana, A., Pinna, L., Letham, D. S., Kato, J., Detivaud, L., Leclerc, S., and Meijer, L. (1994) *Eur. J. Biochem.* **224**, 771–786.
18. Huang, D., Farkas, I., and Roach, P. J. (1996) *Mol. Cell Biol.* **16**, 4357–4365.
19. Schulze-Gahmen, U., Brandsen, J., Jones, H. D., Morgan, D. O., Meijer, L., Vesely, J., and Kim, S.-H. (1995) Proteins Struct. Funct. Genet. **22**, 378–391.

20. Klein, P. S., and Melton, D. A. (1996) *Proc. Natl. Acad. Sci. USA* 93, 8455–8459.
21. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* 135, 397–402.
22. Meggio, F., Donella Deana, A., Ruzzene, M., Brunati, A. M., Cesaro, L., Guerra, B., Meyer, T., Mett, H., Fabbro, D., Furet, P., Dobrowolska, G., and Pinna, L. A. (1995) *Eur. J. Biochem.* 234, 317–322.
23. Meyer, T., Regenass, U., Fabbro, D., Alteri, E., Rosel, J., Muller, M., Caravatti, G., and Matter, A. (1989) *Int. J. Cancer* 43, 851–856.
24. Meijer, L. (1996) *Trends Cell. Biol.* 6, 393–397.
25. Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzuu, Y., and Iba, H. (1987) *J. Antibiotics* 40, 706–708.
26. Segel, I. H. (1975) *in* Enzyme Kinetics—Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, pp. 465–504, Wiley, New York.